Comparison of Tumor-Specific Immunogenicities of Stress-Induced Proteins gp96, hsp90, and hsp70[1]

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Stress-induced proteins (or heat shock proteins (HSPs)) of 96 kDa size (gp96) have been shown previously to elicit specific immunity to tumors from which they are isolated. In this report, we show that in contrast to Meth A-derived gp96, gp96 preparations derived from normal tissues did not elicit immunity to Meth A sarcoma at any dose tested. Further, in light of recent studies showing that other major cellular HSPs hsp90 and hsp70 also elicit tumor-specific immunity, we have compared the relative immunogenicities of gp96, hsp90, and hsp70 derived from the Meth A sarcoma. The proteins gp96 and hsp70 were observed to be highly and equally immunogenic, whereas the immunogenicity of hsp90 was approximately 10% of that of gp96 or hsp70. It is suggested that the poor immunogenicity of hsp90 results from its lack of a measurable ATPase activity, which has been implicated in the ability of HSPs to transfer peptide to acceptor molecules. This is the first study that documents the lack of immunogenicity of gp96 preparations derived from normal tissues and compares the immunogenicity of each of the three major cellular HSPs in one tumor system. *Journal of Immunology*, 1994, 152: 5398.

Immune response to syngeneic or autologous cancer was first demonstrated convincingly in methylcholanthrene-induced sarcomas of inbred mice (1–6). The molecules responsible for the individually distinct immunogenicity of these tumors were identified independently as cell-surface glycoproteins of 96 kDa (gp96) (7) and intracellular proteins of 84 to 86 kDa (8). It was demonstrated that immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors. Initially, the two molecules appeared to be unrelated because of their sizes and because rabbit antisera to each did not cross-react with the other. However, isolation and characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed that gp96 and p84/86 were, respectively, the endoplasmic reticular (ER)[4] and cytosolic counterparts of the same heat shock protein (HSP) (9–11). This observation creates a curious paradox: HSPs are among the most highly conserved proteins in living systems, whereas the immunogenic Ags of chemically induced mouse sarcomas show individually distinct tumor-specific antigenicity. In view of the observation that HSPs bind a variety of molecules, including peptides, it was suggested that gp96 and p84/86 HSPs are not immunogenic per se, but are carriers of antigenic peptides (11, 12). Such peptides were recently demonstrated to be associated with gp96 (13). Further, a well-characterized and major peptide-binding HSP, the hsp70, was shown recently to elicit immunity to the tumor from which it is isolated but not to antigenically distinct tumors (14). It was further shown that an hsp70 preparation depleted of peptides lost its immunogenic activity. Collectively, these observations lend support to our original thesis (11, 12) regarding the role of HSPs in eliciting specific immunity to cancers.

One key question in this emerging paradigm has to do with the immunogenicity of HSPs derived from normal tissues. In case of hsp70, it was demonstrated recently that, whereas the tumor-derived hsp70 preparations are immunogenic, corresponding preparations derived from normal tissues are not (14). This is consistent with the idea that immunogenicity of HSP preparations derives from the altered (e.g., as a result of carcinogen-induced mutations), and hence immunogenic, peptides expected to be present in tumors but not in normal tissues (12). This thesis is further developed in the studies reported here by the

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[4] Abbreviations used in this paper: ER, endoplasmic reticulum; HSP, heat shock protein.

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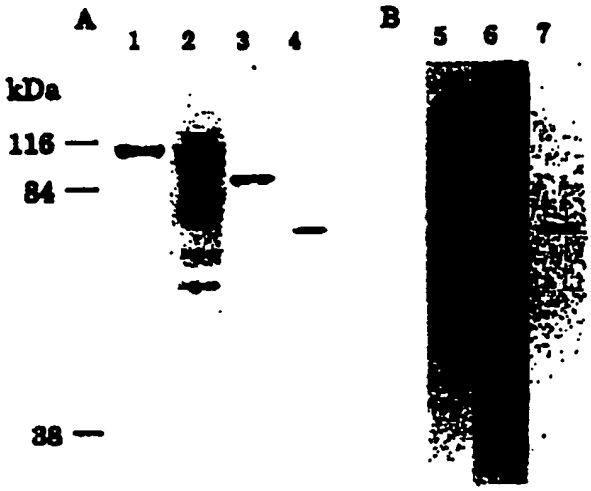

FIGURE 1. Silver-stained SDS-polyacrylamide gels of HSP preparations used in tumor rejection assays. *A*, gp96 (*lane 1*), hsp90 (*lane 3*), and hsp70 (*lane 4*) derived from Meth A sarcoma. *Lane 2* shows gp96 from liver and spleen of normal mice. The gp96 band in this lane constitutes over 98.5% of the total protein, as determined by gel scanning. *B*, Immunoblot of Meth A-derived gp96 (*lane 5*), hsp90 (*lane 6*), and hsp70 (*lane 7*). Abs used in immunoblotting are described in *Materials and Methods*.

demonstration that gp96 preparations isolated from normal tissues are not immunogenic against the Meth A sarcoma, although the same quantity of gp96 isolated from the Meth A sarcoma elicits specific and potent tumor resistance. Furthermore, we compare the relative immunogenicities of gp96, hsp90, and hsp70 derived from the same tumor and observe significant differences among the three. This is the first study that examines the tumor-specific immunogenicities of all major cellular HSPs in a single system using a single assay.

Materials and Methods

Mice, tumors, and Abs

BALB/cJ mice (viral Ag free) were obtained from The Jackson Laboratories (West Grove, PA) and were maintained in the virus-free mouse facilities at Mount Sinai School of Medicine (New York, NY). The tumors Meth A, CMS4, and CMS5 have been described earlier (7, 15). Only the ascites form of Meth A was used in the present study for passage, for challenge as well as for preparation of the different HSPs. Five million ascites cells were used for serial passage of the tumor. Abs to gp96 (anti-GRP94, SPA-850, clone 9G10) and to hsp70 (anti-HSP72/73, SPA-820, clone N27F3-4) were purchased from StressGen (Sidney, BC, Canada). The Ab to hsp90 (anti-HSP90, MA3-011, clone 3G3) was obtained from Affinity BioReagents (Neshanic Station, NJ).

Purification of GP96, HSP90, and HSP70

Proteins gp96, hsp90, and hsp70 were purified simultaneously from Meth A sarcoma cells, by a combination of published methods (7, 13, 14) and some modifications. Briefly, a 50-ml Meth A cell pellet was homogenized in 200 ml of hypotonic buffer (20 mM $NaHCO_3$, 0.5 mM PMSF, pH 7.1) and a 100,000 × *g* supernatant obtained. This was applied to a Con A-Sepharose column. The Con A-bound proteins were used for purification of gp96 as described (7). The Con A-unbound material was used for purification of hsp70 as described (14). For purification of hsp90, Con A-unbound material was dialyzed against 20 mM of sodium phosphate, pH 7.4, 1 mM EDTA, and 250 mM NaCl. This fraction was resolved on the Mono Q Pharmacia FPLC system (Piscataway, New Jersey), equilibrated with 20 mM of sodium phosphate, pH7.4, 1 mM EDTA, and 200 mM NaCl. The proteins were eluted by a 200- to 600-mM NaCl gradient (15). The hsp90-containing fractions identified by immunoblots with anti-hsp90 mAb 3G3 (Affinity BioReagents) were pooled and used for immunization of mice.

SDS-PAGE and protein blotting

Proteins were resolved on SDS-PAGE, electrophoresed, blotted to nitrocellulose, and probed with appropriate Abs, as described (7).

Tumor rejection assays

Mice were immunized by subcutaneous injection of HSP preparations (in 200 μl PBS), given in the nape of the neck. Two immunizations at weekly intervals were given. Mice were challenged by intradermal injections of the indicated number of live tumor cells (in 200 μl PBS) 1 wk after the last immunization. The challenge dose was given in the back of the mouse after a close shave. A high viability of tumor cells (>98%) is an important prerequisite for reproducible results.

Results

Purification of HSPs

Proteins gp96, hsp90, and hsp70 were purified simultaneously from a 50-ml pellet (approximately 10^{10} cells) of Meth A cells, and approximately 600 μg of gp96, 6 mg of hsp90, and 1.8 mg of hsp70 were obtained typically. The yield of gp96 and hsp90 represents a substantial fraction of the total amount present in the cells (>60%), but the recovery of hsp70 was relatively poor (<20%) because of our inability to use ATP-agarose chromatography, routinely used for purification of hsp70 (16). We have observed that exposure of hsp70 to ATP depletes it of its immunogenic activity, by dissociating peptides bound to hsp70 (14).

The gp96 preparations were apparently homogeneous (Fig. 1*A*, *lanes 1* and *2*), and a 110-kDa protein suggested by DeLeo et al. (17) to be the active molecule in gp96 preparations was not observed.[3] Lower m.w. bands resulting from auto-degradation of gp96 (7) were occasionally seen (Fig. 1*A*, *lanes 1* and 2). Preparations of hsp90 were also homogeneous (*lane 3*). Two isoforms of 84 kDa and 86 kDa exist

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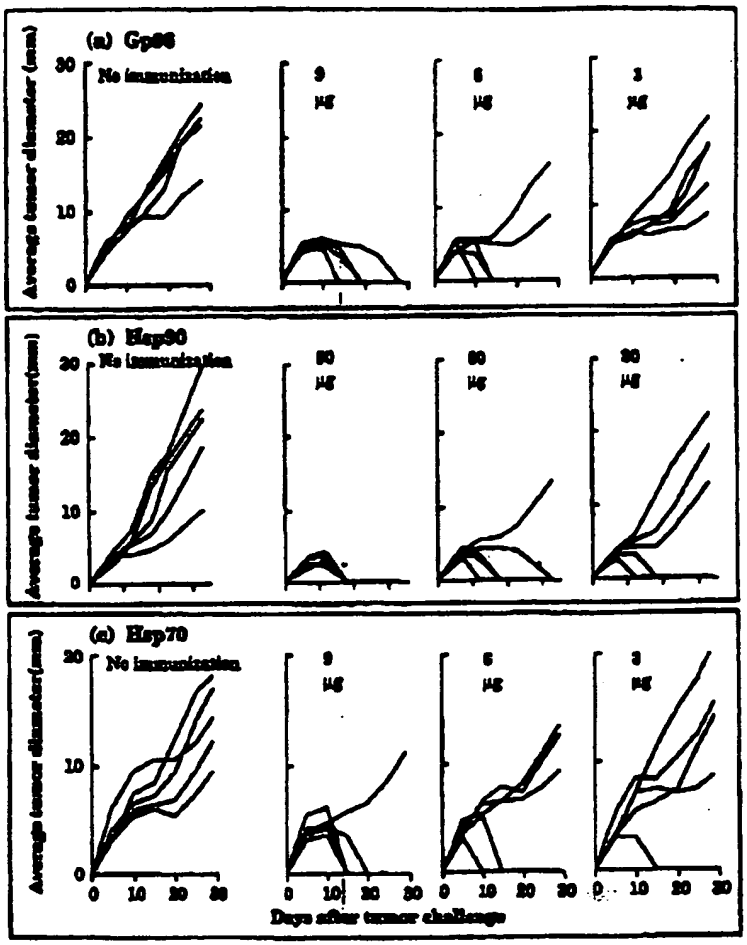

FIGURE 2. Relative immunogenicities of Meth A-derived gp96 (*a*), hsp90 (*b*), and hsp70 (*c*). Groups of mice (five mice per group) were immunized with PBS or varying doses of the HSPs derived from Meth A as shown in Figure 1. Mice were immunized as described in *Materials and Methods* and were challenged with 80,000 Meth A cells intradermally. Each *line* represents the kinetics of tumor growth in one mouse.

of hsp90, and both isoforms are presumed to be present in our preparations. The hsp70 preparations were occasionally, although not always, observed to contain other proteins that did not constitute more than 5% of the total protein. There are seven or more isoforms of hsp70, and our preparations are expected to be mixtures of those. However, the endoplasmic reticular isoform of hsp70, the grp78, or BiP appears not to be present in our preparations, as it is distinguishable from other hsp70 species from its slower migration. The three HSPs were recognized by respective Abs on protein blots (Fig. 1*B*).

Tumor immunity elicited by immunization with HSPs

Groups of mice (five mice per group) were immunized with PBS or with varying quantities of gp96, hsp90, and hsp70 and were challenged with 80,000 Meth A ascites cells (viability >99%) (Fig. 2). All mice that were immunized with PBS developed rapidly growing tumors. In contrast, mice immunized with HSPs were protected from Meth A challenge. The protection was directly dependent on the dose of immunizing HSP; mice immunized with 3 μg (per injection) of gp96 or hsp70 showed slight or no protection from a Meth A challenge, those immunized with 6 μg of gp96 or hsp70 showed protection in about 50% of the mice, whereas 80% to 100% of the mice immunized with 9 μg gp96 or hsp70 were protected (Fig. 2, *a* and *c*). In contrast to the immunogenicity of Meth A-derived gp96 or hsp70, immunization of mice with corresponding doses of Meth A-derived hsp90 did not afford any protection to the 80,000-cell Meth A challenge (data not shown). However, immunization with increased doses of hsp90 was observed to be protective (Fig. 2*b*). A proportion of mice immunized with 30 or 60 μg of hsp90 resisted Meth A challenge, although immunization with 90 μg of hsp90 rendered 100% of the immunized mice resistant to Meth A challenge. Thus, on a protein-quantitative basis, gp96 and hsp70 appear to be equally immunogenic, whereas the immunogenicity of hsp90 is about 10% of that of gp96 or hsp70. To test the specificity of immunogenicity of the three HSPs, mice that had rejected Meth A after immunization with the HSPs were challenged with 50,000

Table I. *Comparison of relative immunogenicity of varying quantities of Meth A-derived gp96 against varying levels of challenges with Meth A cells*[a]

No. of Meth A cells Used for Challenge	Frequency of Tumor Take in			
	Naive Mice	Mice Immunized with Meth A-Derived gp96		
		3 μg	6 μg	9 μg
1×10^6	3/4	4/4	2/5	1/4
8×10^4	5/5	5/5	2/5	0/5
5×10^4	5/5	4/4	0/4	2/5
3×10^4	4/4	4/4	ND[b]	0/4

[a] Mice (4 or 5 mice per group) were immunized with PBS or with indicated quantities of gp96 derived from Meth A sarcoma. Other experimental details are the same as in legend to Table 1.
[b] Not done.

Table II. *Lack of immunogenicity of gp96 derived from normal tissues against Meth A sarcoma*[a]

No. of Meth A cells Used for Challenge	Frequency of Tumor Take in				
	Naive Mice	Mice Immunized with Meth A-Derived gp96	Mice Immunized with gp96 Derived from Normal Tissues		
		6 μg	3 μg	6 μg	9 μg
1×10^5	3/4	2/5	4/4	4/4	4/4
8×10^4	4/4	0/4	4/4	3/4	3/4
5×10^4	4/4	0/4	3/4	3/4	3/4

[a] Mice (4 or 5 mice per group) were immunized with PBS or with 6 μg of gp96 derived from Meth A sarcoma (as a positive control), or with 9, 6, or 3 μg of gp96 derived from liver and spleen. Other experimental details are the same as in legend to Figure 2 except that mice were challenged with 100,000, 80,000, or 50,000 Meth A cells intradermally. Frequency of tumor take is shown as measured on day 30 after tumor challenge.

CMS4 or CMS5 cells. Unimmunized mice were similarly challenged at the same time. As reported earlier (7, 8, 14), no differences were observed in frequency of tumor take of CMS4 and CMS5 among the various groups (data not shown).

To test if there was a correlation between the immunizing dose of gp96 and resistance to different levels of tumor challenge, mice immunized with varying doses of gp96, hsp90, or hsp70 (as in Fig. 2) were challenged with higher (100,000 cells) or lower (50,000 or 30,000) numbers of Meth A cells. It was observed that injection of 9 μg of gp96 (which offered protection against 80,000 Meth A cells in 100% of the immunized mice,) elicited protection in only a proportion (3/5) of mice challenged with 100,000 cells (Table I). The effect of increased dose of gp96 on a 100,000-cell challenge was not tested. Among mice challenged with 50,000 or 30,000 Meth A cells, it was observed that the doses required to elicit protection against the lower challenges were similar to those required to immunize against an 80,000-cell challenge. Smaller doses (such as 3 μg of gp96) did not elicit immunity against smaller challenge, whereas larger doses elicited immunity to all levels of challenge tested (Table II). Two conclusions may be drawn from these observations: 1) administration of 9 μg of gp96 can protect mice against any tumor challenge up to, but not larger than, 80,000 to 100,000 cells; and 2) within this limit of tumor challenge, a certain minimum quantity of immunizing HSP is needed, regardless of the level of tumor challenge. Smaller doses do not necessarily protect against smaller levels of tumor challenge. Similar observations have been made with hsp70.

Immunogenicity of gp96 derived from normal tissues

Protein gp96 was purified from livers and spleens of healthy BALB/cJ mice (Fig. 1A, *lane 2*) and tested for homogeneity by SDS-PAGE and silver staining. The yield of gp96 from normal tissues was comparable to the yield from Meth A cells. Varying quantities of tissue-derived gp96 (3, 6 or 9 μg/injection) were used to immunize mice, as described. In parallel, mice were also immunized with 9 μg of Meth A-derived gp96, and all mice were challenged with 100,000 Meth A cells. It was observed (Table II) that 3 of 5 mice immunized with 9 μg of Meth A gp96 were protected from tumor challenge. In contrast, all mice immunized with any of the three doses of gp96 derived from normal tissues succumbed to tumor challenge. This effect was seen more clearly in mice challenged with 80,000 or 50,000 Meth A cells (Table II); 100% of the mice (4/4 in each group) immunized with 9 μg of Meth A-derived gp96 were protected from tumor challenge, whereas mice immunized with gp96 derived from normal tissues were not protected against tumor challenge to any significant degree. One of four mice immunized with tissue-derived gp96 did resist the lower tumor challenge, but the significance and specificity of this modest protection is not clear. It is conceivable that gp96 elicits some auto-HSP-reactive helper T cells (18), which elaborate lymphokines such as IL-2 that help expand the anti-tumor precursor CTL population. In this case, the slight protective effect of gp96 derived from normal tissues is expected to be nonspecific.

Discussion

Administration of any of the three major cellular HSPs, gp96, hsp90, and hsp70 purified from Meth A cells elicits specific immunity against Meth A but not against antigenically distinct tumors. Immunization of mice with corresponding quantities of gp96 derived from normal tissues does not protect mice against the three levels of Meth A challenge tested. In spite of the tumor-specificity of immunity elicited by tumor-derived HSPs, evidence for tumor-specific mutations has not been found in gp96 or hsp90 cDNAs (11, 19). This led us to suggest that gp96 may not be immunogenic per se, but may be associated with antigenic peptides (11, 12). Recently, peptides were indeed shown to be associated with gp96, and it was further demonstrated that gp96 is an ATP-binding protein and

has an ATPase activity (13). As gp96 is among the most abundant components of the lumen of the ER (20), and peptide-charging of MHC class I occurs in that location (21) in an ATP-dependent manner (22), it was suggested that gp96 acts as a general peptide-acceptor in the ER and facilitates transfer of peptides to MHC class I. The suggestion that the immunogenicity of HSPs derives from associated peptides is also consistent with the observations of Flynn et al. (23, 24) that a broad spectrum of peptides can bind to the ER HSP grp78 or BiP. Furthermore, we have demonstrated recently that hsp70 preparations derived from the Meth A sarcoma lose their immunogenicity after elution of the associated peptides by ATP (14). The corresponding experiment with gp96 is difficult to do because the gp96-peptide complex is not sensitive to treatment with ATP, presumably the result of the differences in regulation of the ATPase activities of the two molecules (13). The acid treatment used for elution of peptides irreversibly denatures the gp96 molecule, such that it loses the ATPase activity. Neither a stripped gp96 preparation, nor the dissociated peptides by themselves are immunogenic (not shown).

The association of HSPs with peptides can occur in a variety of cellular compartments. Gp96 molecules encounter peptides in the lumen of the ER, whereas hsp90 and hsp70 may do so in the cytosol, which is presumed to be the site of generation of antigenic peptides before they are transported to the ER. The peptide-binding property of HSPs may be the common denominator of their tumor-specific immunogenicity. The specificity of immunogenicity of the tumors, and of the HSP preparations derived from them, may be a consequence of the randomness of spontaneous or induced mutations that mediate and accompany malignant transformation, as shown in the tumor-negative system by Boon (25).

A comparison of the relative immunogenicities of gp96, hsp90, and hsp70 showed that although gp96 and hsp70 are equally immunogenic, the immunogenicity of hsp90 is approximately 10% that of gp96 or hsp70 (Fig. 2). This may result from one of three possibilities. There may be a smaller net quantity of associated peptides with hsp90 than with gp96 or hsp70. However, this appears unlikely as all three HSPs are observed to have approximately equal amounts of associated peptides as judged by the reverse-phase HPLC profiles of peptides (13, 14, and H. Udono and P. K. Srivastava, unpublished observations). Alternatively, hsp70 and gp96 may have access to a broader, more diverse pool of antigenic peptides than hsp90. Hsp70 is localized in the endosomes, nuclei, and plasma membranes apart from the cytosol (26), and gp96 is localized in the ER, which is the transit point for all antigenic peptides (21). The intracellular localization of hsp90 is less well understood. As a third possibility, differences between gp96 and hsp70 on one hand and hsp90 on the other may derive from the fact that gp96 and hsp70 are ATPases, although hsp90 is not (13). It is conceivable that the peptide-binding and peptide-transfer capacities of hsp90 may be abbreviated as a result of the lack of an ATPase activity. We favor the third possibility.

Our results do not address the question of the immunologic circuitry by which administration of soluble HSPs leads to a specific cellular anti-tumor immune response. We suggest that peptides are transferred from the HSP to MHC class I either directly or through internalization of the HSP-peptide complex, and that this process occurs in specialized Ag-presenting cells (27, 28). Cytotoxic T lymphocyte precursors then recognize the MHC Class I-peptide complexes on the presenting cells and are activated. A certain threshold of the number of presenting cells that display the transferred peptides with MHC class I appears to be essential for an effective protection from tumor challenge, as suggested by our observation that the optimal quantity of immunizing HSP preparation does not decrease with lower challenge levels.

Studies on the role of HSPs in immune response in other systems have uncovered three major paradigms: 1) where HSPs are processed and presented like other Ags by MHC class I or class II molecules and recognized by $\alpha\beta$ T cells (29–31), 2) where $\gamma\delta$ T cells recognize HSPs by a poorly understood mechanism (32, 33), and 3) where an Ab response is elicited against a specific HSP of the host (an autoimmune response) or an infectious agent (34, 35). Our studies, which suggest that immunization with HSPs may elicit a specific cellular immune response not against the HSPs per se, but against antigenic peptides chaperoned by them, represent a new, fourth, paradigm in immune response to HSPs.

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